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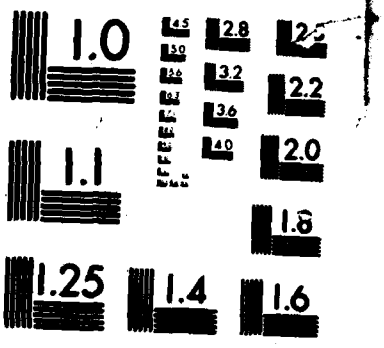
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Immunoglobulin molecules such as IgG recognize an antigenic determinant (epitope) by means of binding sites located in the variable region of the antibody called the antigen-binding site or idiotype. It is possible to produce anti-idiotypic (anti-Id) antibodies that recognize and bind to this site and that in turn contain an "internal image" of the antigen. These antibodies thus resemble the actual antigen and offer a means of immunizing against specific epitopes (for example, a virus antigen) without the presence of the actual virus. To

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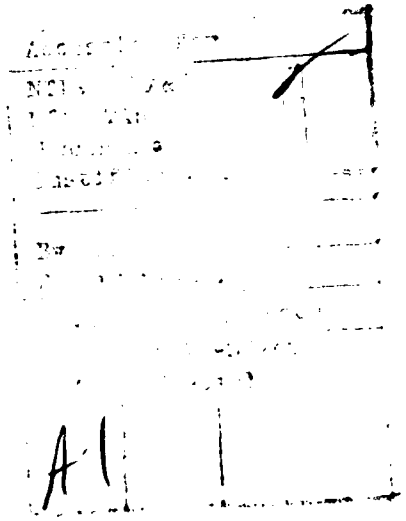
determine if anti-Id antibodies could be developed and used to induce an immune response against herpes simplex virus, monoclonal antibodies were first produced aganist the type 1 (HSV-1) and the type 2 (HSV-2) viruses. Using several techniques, these antibodies were then injected into mice and rabbits to produce both monoclonal and polyclonal antibodies. After screening, the polyclonal anti-Id antibodies were found to be most active and were used to immunize mice in attempts to induce the formation of anti-HSV antibodies. The results indicated that there was weak response of the rabbit serum towards homogous virus-infected cells. The results also indicate the feasibility of this type of research and points towards additional experiments to optimize the experimental prodecures used.



N00014-84-C-0433
Ernest D. Marquez

FINAL REPORT
OFFICE OF NAVAL RESEARCH -IMMUNOLOGY PROGRAM
N00014-84-C-0433
IMMUNIZATION AGAINST INFECTION USING ANTI-IDIOTYPIC AB
ERNEST D. MARQUEZ, PRINCIPAL INVESTIGATOR

1.0 Introduction

Immunoglobulin molecules such as IgG recognize an antigenic determinant by means of binding sites located in the variable region of the antibody called the antigen-binding site or idiotype. It is possible to produce anti-idiotypic antibodies that recognize and bind to this site and that in turn contain an "internal image" of the antigen. These antibodies thus resemble the actual antigen and offer a means of immunizing against specific epitopes (for example, virus antigens) without the presence of the actual virus. A number of investigators have demonstrated the feasibility of this procedure although protection against a complex virus such as herpes simplex virus (HSV) has not been demonstrated. This proposal was designed to determine whether protection against infection by HSV types 1 or 2 can be induced using anti-idiotypic antibodies.

2.0 Experimental Objectives

The overall objective of this work was to produce anti-idiotypic (anti-Id) antibodies and to determine if these anti-Id antibodies could be used to protect animals against infection by herpes simplex virus (HSV). In particular, we wished to develop anti-Id antibodies using anti-HSV monoclonal antibodies as the templates. After screening for the presence of anti-Id antibodies, they would be tested in vivo to determine if the antibodies could elicit production of anti-HSV antibodies and, in a separate set of experiments, determine if the anti-Id antibodies could elicit cell-mediated immunity against HSV-infected cells. Finally, the anti-Id antibodies would be tested in vivo to determine if they could elicit an immune response and therefore protect the animal against infection by HSV.

2.0 Detailed Final Report

2.1 Production of monoclonal antibodies against HSV-1 and HSV-2

In the early part of the work it was decided to produce more monoclonal antibodies against HSV Type 1 and Type 2. To do this, mouse fibroblast cells previously infected with HSV-1 or HSV-2 were inactivated using ultraviolet light and injected into mice for production of monoclonal antibodies. For this purpose, a unique combination of myeloma and mouse was used. The myeloma used was the FOX-NY cell line having a lesion in the locus for adenine phosphoribosyl transferase (APRT) in addition to the conventional HPRT deletion. Using this myeloma, those hybridomas that produce antibodies but lose the X chromosome (HPRT) and would die in HAT medium would live in the selection medium AAT (adenine,

aminopterin, thymidine). In addition, the mouse that was immunized for the fusion was the RBF/Dn strain that has a translocation between chromosomes 8 (APRT) and 12 (Ig Heavy Chain). A hybrid in AAT selection medium would die if antibody production was lost due to loss of chromosome 12. The net result of using this combination was that the number of hybrids was much higher than with conventional fusions using the BALB/c mouse and, for example, the NS-1 myeloma cell as shown below.

COMPARISON OF FUSION EFFICIENCY
FOX-NY SYSTEM VERSUS NS-1 SYSTEM

SYSTEM	AVERAGE NO. OF HYBRID CLONES PER FUSION
BALB/c + NS-1	300-600
RBF/Dn + FOX-NY	900-1500

The screening tests for monoclonal antibodies to be included in this study were carried out using the following procedures: To test for specificity against HSV, modified ELISA plates were set up. Antigens from HSV-1, HSV-2, and CMV-infected (control) cells were attached to separate wells of 96-well plates using standard methods. Supernatants from hybridoma cells were then reacted with each of the three antigen plates. Those that reacted with HSV-1 or HSV-2 exclusively were selected for further testing.

The next test was for the ability to bind to protein A of Staphylococcus aureus. To carry out this screen, supernatants from positive wells were placed in contact with IgSorb, a commercially available Protein A suspension (inactivated and fixed S. aureus cells). After adsorption had occured, the presence of the bound antibody was determined using fluorescein-labeled anti-mouse IgG from goat [F(Ab)2 fragments]. Those clones of hybridoma cells producing antibody that were specific for HSV-1, HSV-2, or both and that bound to Protein A were selected for inclusion in this study and are shown below:

SELECTION OF ANTI-HSV HYBRIDOMAS

CLONE	SPECIFICITY	PROTEIN A BINDING
4E11	HSV-1	+
2C1	HSV-1	+
gE	HSV-2	+
gF	HSV-2	+
4D1	HSV-1,2	+

2.2 Production of anti-Id antibodies

Attempts were made to produce anti-Id antibodies using both monoclonal and polyclonal antibody techniques. In both cases, the anti-HSV antibodies, the immunogen, were presented as either purified antibodies or as antibodies bound to Protein A.

2.2.1 Production of polyclonal anti-Id antibodies

Purified anti-HSV monoclonal antibodies were produced by first precipitating the antibody from culture supernatants using 50% saturated ammonium sulfate. Final purification was achieved using affinity chromatography on a Sepharose-Protein A column. The antibodies were eluted using a step-wise pH gradient, neutralized and dialysed against phosphate buffered saline (PBS). After determining that the antibodies were still reactive and determining the protein concentration, the monoclonal antibodies were mixed with Freund's complete adjuvant (1:1) and inoculated into BALB/c mice and rabbits. Aliquots containing 100-200 ug of protein were inoculated on days 1, 3, 10, and 21 and specimens of blood taken by retro-orbital puncture after 25 days for determination of anti-Id presence and titer. Thereafter, weekly booster immunizations were administered if a negligible anti-Id titer were detected.

Protein-A-bound anti-HSV antibodies were produced by reaction of hybridoma culture supernatants with Sepharose-Protein A beads until saturation of the beads with antibody had occured. This was tested by using an anti-mouse IgG [F(AB)2] antibody labeled with fluorescein. The beads were washed in PBS containing 0.01% Tween 20 followed by several washes in sterile PBS. After a final test using anti-mouse antibody to determine the presence of the bound anti-HSV antibodies, the beads were inoculated into mice and rabbits using the immunization scheme described above.

2.2.2 Production of monoclonal anti-Id antibodies

For production of monoclonal antibodies, immunization schemes using purified anti-HSV antibodies and antibodies bound to Protein A were used as described above for polyclonal antibodies. In addition, hybridoma cells producing antibodies of the specific idiotype were also used as the immunogen. In this case, the cells contain receptor molecules on their surfaces (monomeric IgM) that should possess the same idiotypes as the IgG molecules that are produced and shed. Using this rationale, hybridoma cells that were fixed in 40% acetone/60% methanol for 5 min at 0 C. All the above immunogens were inoculated into RBF/Dn mice using the immunization scheme described for polyclonal antibodies. The splenocytes from immunized mice were fused with FOX-NY myeloma cells using standard procedures and selection for hybrids allowed to occur in media (AAT).

2.3 Purification of polyclonal anti-Id antibodies

Purification of both mouse and rabbit anti-Id antibodies was carried out using a two stage procedure. In the first stage, the antisera was partially purified by precipitation of the immunoglobulins using (50%) saturated ammonium sulfate. After dialysis the immunoglobulins were passed over an immunoaffinity column containing covalently bound normal mouse IgG (i.e. the idiotype for HSV was not present). The antibodies were repeatedly passed over the column until minimal amounts of immunoglobulin bound to the column.

In the second stage, the antibodies that did not bind to the first column (anti-Id antibodies) were first passaged through a column

containing monoclonal antibodies possessing a different idiotype (cytomegalovirus). Those antibodies that did not bind were next adsorbed onto a column of covalently bound monoclonal antibodies containing the homologous idiotype. The bound antibodies were then eluted from the column using a pH gradient and dialysed against PBS.

2.4 Screening tests for the presence of anti-Id antibodies were carried out in two steps for monoclonal as well as polyclonal antibody preparations. The first step was a broad screening procedure involving an enzyme linked immunoassay (ELISA) while the second was a more specific competitive assay.

2.4.1 ELISA screening test for anti-Id antibodies

In this screening procedure, two wells were used for each sample to be tested. The first contained bound anti-HSV monoclonal antibody containing the idiotype of interest and the second well contained a monoclonal antibody possessing an unrelated idiotype (anti-CMV). after blocking the wells to prevent nonspecific binding, the putative anti-Id antibody was added and incubated for one hour. After washing, the wells were evacuated, washed and anti-mouse IgG (labeled with horseradish peroxidase) added. Those antibody preparations that reacted with wells containing the idiotype and not with the unrelated idiotype were retained.

2.4.2 Competitive screening procedure for anti-Id antibodies

In this procedure, polyacrolein beads (Polysciences, Inc.) were used to covalently bind HSV antigens. The beads were then washed, blocked with 3% BSA and resuspended to a final volume of 10% (W/V). Twenty microliters of bead suspension were then added to wells of a Millititer 96-well plate.

To determine the concentration of anti-HSV monoclonal antibody to be used in the assay, increasing dilutions of antibody (50ul) were added to wells containing the beads. After incubation for 60 min with continuous shaking, the antibody dilutions were removed by vacuum filtration and the beads washed 4 times using 200 ul of PBS containing 0.05% Tween 20. To each well was then added 50 ul of anti-mouse IgG labeled with horseradish peroxidase (HRP). After incubation for 60 min, the beads were again washed and 200 ul of HRP substrate (Ngo-Lenhoff) were added. The absorbance readings for each well were made by transferring the contents of the wells to a regular 96-well plate and taking readings using a Flow Laboratories plate reader.

The dilution of antibody considered to be best suited for the anti-Id assay was that at which a slight inhibition of antibody reactivity resulted in a significant decrease in absorbance. Below is shown a typical set of results.

TITRATION OF ANTI-HSV ANTIBODIES USING BEAD ASSAY

	RECIPROCAL OF DILUTIONS					
ANTIBODY	20	40	80	160	320	640
gE	.901	.895	.890	.790	.303	.106
gF	.890	.901	.805	.740	.402	.280
4E11	.780	.806	.765	.620	.398	.106

Based on these assays, after correction for background, the optimal dilution of all three antibodies as used in tests would be approximately 1/320.

To determine the presence of anti-Id antibodies in hybridoma supernatants, 50 ul of anti-HSV antibodies were incubated with 50 ul of putative anti-Id antibodies in a separate tube for one hour. After incubation, the mixture was added to the beads in the Millititer plates and incubated for an additional hour. After incubation the beads were washed 4 times using PBS + 0.05% Tween 20 and reacted with anti-mouse IgG for 1 hour. After incubation the beads were again washed and substrate for HRP added and read using a plate reader. Readings from wells containing putative anti-Id antibodies were compared to those containing negative control supernatants.

The results of these screening procedures are shown below.

RESULTS OF SCREENING ASSAYS
FOR MONOCLONAL ANTI-ID ANTIBODIES

	NUMBER OF POSITIVE CLONE SUPERNATANTS*	
ANTI-HSV IDIOTYPES	ELISA	BEAD ASSAY
4E11	6	0
2C1	8	0
gE	4	0
gF	2	0
4D1	6	0

* POSITIVE for HSV idiotype and negative for CMV idiotype

RESULTS OF SCREENING ASSAYS
FOR POLYCLONAL ANTI-ID antibodies

ANTI-HSV IDIOTYPES	ELISA	BEAD ASSAY
4E11		
MOUSE ANTIBODY	+	+/-
RABBIT ANTIBODY	+	+/-
2C1		
MOUSE ANTIBODY	+	+
RABBIT ANTIBODY	+	+
gF		
MOUSE ANTIBODY	+	+/-
RABBIT ANTIBODY	+	+
gE		
MOUSE ANTIBODY	+	+/-
RABBIT ANTIBODY	+	+/-
4D1		
MOUSE ANTIBODY	+	+/-
RABBIT ANTIBODY	+	+/-

The results of these screening assays indicated that the polyclonal anti-Id antibodies were the only ones that could be considered for further testing. The monoclonal anti-Id antibodies that were screened were either not specific for the HSV idiotype or their avidity was so low that they could not withstand the washing procedures inherent in the confirmation assays. Since approximately one half of the total hybridoma clones were tested (the rest being frozen in liquid nitrogen) it is possible that some specific monoclonal anti-Id antibody producing clones exist but time does not permit a completion of the screening process for these.

The polyclonal antibody preparations that were chosen for further study included anti-Id antibodies against:

1. Anti-4E11 (HSV-1) idiotype (Rabbit Antibody)
2. Anti-2C1 (HSV-1) idiotype (Mouse Antibody)
3. Anti 2C1 (HSV-1) idiotype (Rabbit Antibody)
4. Anti-gF (HSV-2) idiotype (Rabbit Antibody)

2.5 Induction of anti-HSV antibodies using anti-Id antibodies

Because of limitations imposed by time and animal-facility constraints, one standard protocol was used for immunization of mice with the anti-Id

antibodies. Mice (in triplicate) received immunizations of 100-200 ug of each antibody above on days 1, 3, 10, and 21. All immunizations were carried out in the presence of Freund's complete or incomplete adjuvant. On day 25, the mice were sacrificed and the serum from these animals tested for the presence of anti-HSV antibodies using HSV-infected cells on coverslips as the antigen. The presence of anti-HSV antibody was disclosed by means of a second antibody (anti-mouse IgG) labeled with fluorescein. As controls, HSV-1 and HSV-2 infected cells were stained with monoclonal antibodies directed against HSV-1 or HSV-2 directly labeled with fluorescein (Syva Corporation). The results of these preliminary experiments are shown below:

INDUCTION OF ANTI-HSV ANTIBODIES
USING ANTI-ID ANTIBODIES

	Reactivity of Induced Anti-HSV Antibodies		
Anti-Idiotypic Antibody Used for Induction	HSV-1 Antigens	HSV-2 Antigens	Uninfected Cells
Anti-4E11 idiotype (Rabbit)	+	0	0
Anti-2C1 idiotype (Mouse)	+/-	0	0
Anti-2C1 idiotype (Rabbit)	+	0	0
Anti-gF idiotype (Rabbit)	0	+	0
Anti-HSV-1 (Control)	++++	0	0
Anti-HSV-2 (Control)	0	++++	0

*1 Fluorescence Results: 0, No Fluorescence; +, Weak fluorescence; ++, Moderate fluorescence; +++, Strong fluorescence; ++++, Brightest fluorescence.

The results indicated that the fluorescence seen with antibodies induced by the anti-Id antibodies was either weak or very difficult to see as compared to background. One explanation for these findings is that the immunization scheme is not optimal for induction of anti-Id antibodies. In this case, a variety of immunization schemes should be explored. Another explanation is that the particular antibody subclasses found in either the monoclonal antibody preparations or in the polyclonal antibodies were not the right type. Previous investigators have found that particular subclasses of anti-Id antibodies actually suppress production of antibodies specific for the idiotype. In this case, particular subclasses of anti-Id monoclonal antibodies can be developed or particular subclasses of anti-Id polyclonal antibodies can be isolated using affinity chromatography and used to induce idiotype-specific antibodies.

In summary, these experiments have shown the feasibility of producing anti-HSV antibodies in vivo using anti-Id antibodies as the immunogen. Because of time restraints and budget considerations, the additional experiments required to define and optimize the experimental procedures cannot be done under this contract.

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